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To the Graduate Council:

I am submitting herewith a thesis written by Ciara Diane Csanadi entitled "The Effects of Leucine on Fatty Acid Oxidation in Chronically Active Males." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Nutrition.

Michael B. Zemel, Major Professor

We have read this thesis and recommend its acceptance:

Melissa Hansen-Petrik, Dixie Lee Thompson

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

The Effects of Leucine on Fatty Acid Oxidation in Chronically Active Males

A Thesis
Presented for the
Masters of Science Degree
University of Tennessee, Knoxville

Ciara Diane Csanadi
May 2012

Abstract

Leucine is a branched-chain amino acid that is known to stimulate muscle protein synthesis and prevent muscle protein degradation (22). However, the effects of leucine on fat oxidation are still being determined. Thus, we investigated the effect of leucine on fat oxidation in chronically active males, following a similar study conducted by our laboratory on leucine and fat oxidation in sedentary, overweight subjects. Participants in this study were males, ages 25-55 years old, with BMI values of 18.5-27 kg/m², and participated in >180 minutes/week of vigorous-intensity physical activity. Participants were randomized to receive either a leucine-supplemented beverage or a placebo beverage three times/day for seven days, followed by a seven-day washout period, and then followed by a final seven days on the remaining treatment (leucine or placebo). Leucine supplementation totaled 2.25g/day. Each participant came in after a 12-hour fast and 24-hour abstention from exercise and his resting metabolic rate was obtained using a metabolic cart. Each participant provided 24 hours of collected urine for urea nitrogen analysis and calculation of respiratory quotient and substrate oxidation.

Respiratory quotient and fat oxidation did not change in response to placebo compared to baseline measurements. However, during the leucine treatment, respiratory quotient significantly decreased from 0.85 ± 0.032 to 0.81 ± 0.032 ($p=0.000056$). Fat oxidation during the leucine treatment also increased, but did not reach statistical significance (48 ± 13.19 g/day before treatment and 58.8 ± 20.14 g/day after treatment). Carbohydrate oxidation also exhibited a non-significant decrease during the leucine treatment from 118.6 ± 31.96 g/day to 75.8 ± 38.02 g/day. Our results indicate that leucine decreases the respiratory quotient and may increase fat oxidation in chronically

active men when compared to a placebo.

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Introduction

Over 60% of adults in the United States are classified as overweight or obese. Co-morbid conditions secondary to obesity include hypertension, stroke, diabetes, coronary heart disease, cancer, osteoarthritis, sleep apnea, reduced fertility and depression (1). Factors related to the development of obesity include a decrease in energy expenditure and an increase in energy intake. According to the Centers for Disease Control and Prevention (CDC), in 2007, only 48.8% of Americans participated in moderate-intensity physical activity for at least 30 minutes, 5 or more days a week, and 13.5% were inactive, participating in less than 10 minutes of physical activity per week (2,3).

Extensive research has been conducted in the search for nutritional food components to enhance fatty acid (FA) oxidation and improve weight management efforts. These nutritional aids do not affect energy balance, but can potentially alter fat breakdown. Potential nutritional aids include: epigallocatechin gallate (EGCG) found in green tea, capsaicin found in hot red pepper, and conjugated linoleic acid (CLA) found in beef, milk and milk products (9, 13, 16). Many studies have looked at EGCG and its effects on FA oxidation and ability to prevent obesity in mice (6-8) however its effects in humans are not well studied. Epigallocatechin gallate is one of many polyphenolic flavonoids, known as catechins, found in green tea and is thought to be the most pharmacologically active (9). Several experimental studies have found that chronic consumption of green tea extract (GTE) can increase FA oxidation and prevent obesity in mice (6-9). Human studies have been less conclusive due to small sample sizes and the need for broader ranges of age and body mass index (BMI) to define the optimum dose

(10). Lastly, GTE used in experimental studies in humans is often combined with caffeine, making it hard to determine the true effect of the GTE on FA oxidation.

Another natural herbal supplement studied as a potential weight-loss agent is capsaicin. Capsaicin is the pungent principle of hot red pepper, however there are also non-pungent capsaicin-related substances, called capsinoids. The effects of these two substances on FA oxidation and weight maintenance have been evaluated. It has been found that capsinoids suppress body fat accumulation in mice (11); however, the efficacy of capsinoids in FA oxidation in humans is inconclusive (12). Capsinoids have been found to be safe and easily tolerated when taken orally (12). Capsaicin, however, has such a strong pungency that not all people can tolerate it without negative side effects, despite evidence that it slightly increases FA oxidation when compared to a placebo (13).

Conjugated linoleic acid includes unsaturated fatty acids with 18 carbon atoms and two conjugated double bonds. The two main CLA isomers, trans-10-cis-12 and cis-9-trans-11, have been extensively studied and have been found to have many potential health benefits in animals, such as reducing body fat mass, improving immunological function, preventing atherosclerosis, and modulating cancer and diabetes (14). Cis-9-trans-11 is the main dietary form of CLA, which enhances growth and seems to have an anticarcinogenic effect, whereas trans-10-cis-12 affects lipid metabolism and alters body composition by reducing body weight and fat percentage (19, 20). Conjugated linoleic acid has been studied at length in rodents and has been shown to reduce body fat; however studies in humans have been inconclusive in determining an effect on body composition (14). Whereas some studies in humans have supported the effects of CLA on body fat reduction (79, 80), others have found no significant difference in body

composition between CLA and placebo (78). The proposed reasons for the inconsistent results in human studies are the dose and isomer of CLA used, diet composition in the study, duration, physical activity in the study, genetic disposition to fat accumulation, techniques used to evaluate body composition, and age and maturity of subjects (15, 20). To date, the effectiveness of CLA on humans is controversial and it is not advised to extrapolate to humans the results that have been found in animal studies (21).

The most recent nutritional aid evaluated to potentially increase FA oxidation and contribute to weight management is leucine. Leucine is a branched-chain amino acid that stimulates muscle protein synthesis and decreases muscle protein degradation (56). Recent data from our laboratory demonstrate that leucine promotes energy partitioning from adipocytes to muscle cells in mice, resulting in decreased energy storage in adipocytes and increasing FA utilization in muscle (17). Moreover, a clinical study of the effects of leucine demonstrated that high concentrations of leucine (2.25 g/day) led to a significant increase in FA oxidation in overweight and obese subjects (18). However, the impact of this leucine dosage on FA oxidation in chronically active males has not been determined, and it is not clear whether leucine will exert the same effect in individuals with activity-induced increases in FA oxidation.

Specific Aims

This study is designed to determine the effects of leucine supplementation on fatty acid oxidation in chronically active males (those who undergo at least 3 hours, or 180 minutes, per week of vigorous-intensity physical activity, either muscle-strengthening or aerobic activity or a combination of both).

Literature Review

Mitochondrial Biogenesis

Mitochondrial biogenesis is the making of new mitochondria through the growth and division of pre-existing mitochondria, which are cellular organelles that meet the oxidative energy demands of mammalian organisms (23, 24). Mitochondrial biogenesis is controlled and upregulated by multiple pathways. Metabolic demands, such as fasting, cold exposure and stress, along with physical exercise serve to upregulate mitochondrial biogenesis (25). At the center of these pathways is peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α), a transcriptional coactivator involved in the control of cellular energy metabolism, lipid metabolism and insulin sensitivity (26). PGC-1 α is the master regulator of mitochondrial biogenesis and is induced by exercise and nutritional factors. When PGC-1 α is activated, it coordinates the activity of transcription factors, including nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2), as well as mitochondrial transcription factor A (Tfam). The nuclear respiratory factors activate the transcription of nuclear genes that encode for the mitochondrial respiratory chain proteins. Tfam regulates the transcription of the mitochondrial DNA (27). Overall, PGC-1 α and its coactivators coordinate the activation of 37 mitochondrial genes and 1000 nuclear genes, along with the proliferation and transcription of the mitochondrial genome (25).

PGC-1 α has been at the center of understanding the molecular processes regulating energy metabolism since its discovery over a decade ago (26). Research studies have both knocked out PGC-1 α in mice as well as over-expressed PGC-1 α in rats in efforts to explain its role in substrate metabolism and mitochondrial biogenesis. Leone

et al. (28) conducted a study on PGC-1 α null mice, where they bred PGC-1 α heterozygous mice to generate PGC-1 α null offspring. This study found that organs with high mitochondrial energy demands, such as the heart and slow-twitch muscle, were underdeveloped and weighed less in null mice than those found in wild type control mice. The PGC-1 α null mice were found to develop abnormal body fat and were less susceptible to diet-induced insulin resistance than the wild type controls. Mitochondrial number and respiratory capacity were diminished in the slow-twitch skeletal muscle of the null mice, leading to a reduced muscle performance and exercise capacity. Lastly, this study showed the PGC-1 α null mice were unable to maintain core body temperature following exposure to cold (28). In comparison, Benton et al. (26) conducted a study on the over-expression of PGC-1 α in rats within normal physiological limits. This 24% modest over-expression of PGC-1 α increased mitochondrial biogenesis, fatty acid oxidation, and insulin sensitivity. This amount of upregulation of PGC-1 α is the same as that which can be observed during a single bout of exercise and is sufficient to reprogram the metabolic capacity of skeletal muscle (26).

AMP-activated protein kinase (AMPK) is an energy-sensing fuel provider and another regulator of mitochondrial biogenesis. AMPK activity increases during fasting or exercise when the AMP/ATP ratio increases. When AMPK is activated, it upregulates PGC-1 α expression in skeletal muscle, and mitochondrial biogenesis is increased. AMPK also activates catabolic processes such as fatty acid oxidation and represses anabolic processes such as fatty acid synthesis (29, 30).

Research on AMPK has shown its direct upregulation by exercise. Atherton et al. (31) conducted a study using low-frequency electrical stimulation to mimic endurance

exercise and looked at AMPK activation and PGC-1 α expression. In this study, muscles were isolated from rats and were stimulated for 3 hours a day, 5 days a week for 3 weeks. The results showed that both AMPK phosphorylation and PGC-1 α expression both increased significantly (31). Terada et al. (32) looked at the effects of low-intensity prolonged exercise on PGC-1 α in rat epitrochlearis muscle. Following a 6-hour bout of swimming, PGC-1 α increased significantly. In this study, Terada et al. (32) also incubated rat epitrochlearis muscle for 18-hours with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), which is an AMPK-activator. AICAR is an exercise mimetic, which mimics and potentiates the effects of exercise in efforts to treat metabolic diseases. Following this incubation, PGC-1 α mRNA increased two times higher than the control muscle, showing that AMPK activation during exercise may be involved in the elevated expression of PGC-1 α after exercise (32).

A second energy sensor is sirtuin 1 (Silent Information Regulator 1) (SIRT1), also a regulator of mitochondrial biogenesis. SIRT1 is a NAD⁺-dependent deacetylase, meaning it is activated by energy restriction or an increase in the NAD⁺/NADH ratio. SIRT1 links mitochondrial activity and transcriptional regulation by activating PGC-1 α . Energy depletion is sensed by AMPK and SIRT1, resulting in increased PGC-1 α transcription (76). SIRT1 can be activated by resveratrol, which is a polyphenol found in grapes and red wine and appears to be a mimetic of calorie restriction (27, 34). Csiszar et al. (34) showed resveratrol treatment of human coronary arterial endothelial cells increased mitochondrial mass, upregulated protein expression of SIRT1 and increased SIRT1 activity and PGC-1 α , Tfam and NRF-1 expression in endothelial cells. SIRT1 can also be activated by AMPK. Canto et al. (35) conducted a study to determine how

AMPK acutely increases the NAD^+/NADH ratio and indirectly activates SIRT1. They used C2C12 myotubes and found that AICAR and activation of AMPK by metformin, increased the NAD^+/NADH ratio. This increased NAD^+/NADH ratio was perfectly correlated with PGC-1 α deacetylation, supporting the hypothesis that changes in NAD^+ levels translate AMPK effects onto SIRT1 activity (35).

Calcinuerin (CaN), a calcium-dependent protein phosphatase, and calcium-calmodulin-dependent kinase (CaMK) are two pathways upregulated by the flux of intracellular calcium during exercise, which have been found to induce PGC-1 α and mitochondrial biogenesis. These regulators are only activated by exercise, when muscle contraction generates powerful calcium fluxes (24, 25, 33). Numerous studies have shown that caffeine treatment of isolated rat epitrochlearis muscle, in vitro tetanic muscle contractions, and whole-body exercise in humans all increase the phosphorylation and activation of CAMK (36). Wu et al. (37) found that skeletal muscles from transgenic mice over-expressing active CAMK, showed increases in mitochondrial DNA replication and mitochondrial enzymes involved in FA metabolism, along with a reduction in fatigue development during repeated muscle contractions. It was also found that the over-expression of CaN resulted in the increase of PGC-1 α expression in skeletal muscle (38) and the induction of mitochondrial biogenesis in cardiac myocytes (39).

Exercise also upregulates p38 mitogen-activated protein kinase (p38 MAPK). p38 MAPK directly phosphorylates PGC-1 α , thereby increasing mitochondrial biogenesis (25); in addition, PGC-1 α phosphorylation by p38 MAPK protects against protein degradation, increasing the protein half-life (40). p38 MAPK lies downstream of CAMK in the signaling pathway, therefore an increase in calcium leads to activation of

p38 MAPK. Wright et al. (41) found that by inhibiting CAMK, they could prevent the phosphorylation of p38 MAPK when cytosolic calcium was raised. Therefore, prevention of p38 activation completely blocked the calcium-induced increase in the expression of PGC-1 α .

Leucine and protein synthesis

Skeletal muscle undergoes constant change throughout the day through the synthesis of new proteins and the breakdown of existing proteins. Leucine, a branched chain amino acid (BCAA), has been shown to stimulate protein synthesis in skeletal muscle and inhibit protein degradation. It is unique among amino acids for its role in the translational control of protein synthesis. Leucine-rich foods include (expressed as mg leucine/g protein): dry milk (35.4 mg/g), cheese (29.6 mg/g), beef (28.7 mg/g), chicken (25.1 mg/g), salmon (22.2 mg/g), almonds (14.9 mg/g), soybeans (13.6 mg/g), cottage cheese (12.7 mg/g), eggs (11.7 mg/g), milk (3.75 mg/g) and yogurt (4.9 mg/g) (90, 101). Milk proteins, casein and whey, contain more leucine (mg/g protein) than soy protein. Whey contains 107.5 mg leucine/g protein, casein contains 82.2 mg leucine/g protein and soy contains 81.0 mg leucine/g protein (100). The Recommended Dietary Allowance (RDA) for leucine is 16 mg/kg/day for adults. Leucine is a modulator of the insulin phosphoinositol 3-kinase (PI3-kinase) signal cascade, a nitrogen donor for muscle production, and a critical regulator of translation initiation of protein synthesis (48). The anabolic effect of leucine on protein synthesis is of interest mainly in the maintenance and enhancement of lean body mass. However, leucine also plays a role in reducing the loss of lean body mass in various disease states (51). The mechanisms by which leucine increases protein synthesis have only begun to be understood since the turn of the

century, although it has been known for ~40 years that leucine mediates most of the effects of protein and amino acid intake on protein metabolism (86, 87). There are two signaling pathways through which leucine increases protein synthesis that have been identified: an insulin-dependent and an insulin-independent mammalian target of rapamycin (mTOR) pathway. Leucine directly stimulates mTOR independently of insulin, but also stimulates insulin release, which, in turn, stimulates mTOR via PI3-kinase (87). In the insulin-dependent pathway, the anabolic actions of leucine and insulin activate independent intracellular-signaling pathways that then converge on the mTOR pathway to increase translation initiation and elongation of protein synthesis (42). Through the mTOR pathway, leucine stimulates protein synthesis by enhancing the association of eukaryotic initiation factor 4E (eIF4E) with eukaryotic initiation factor 4G (eIF4G) to form the eukaryotic initiation factor 4F (eIF4F) complex, or by phosphorylating ribosomal protein S6 kinase 1 (S6K1) (Figure 2). Before the eIF4F complex can be formed, eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) must be phosphorylated to release it from eIF4E.

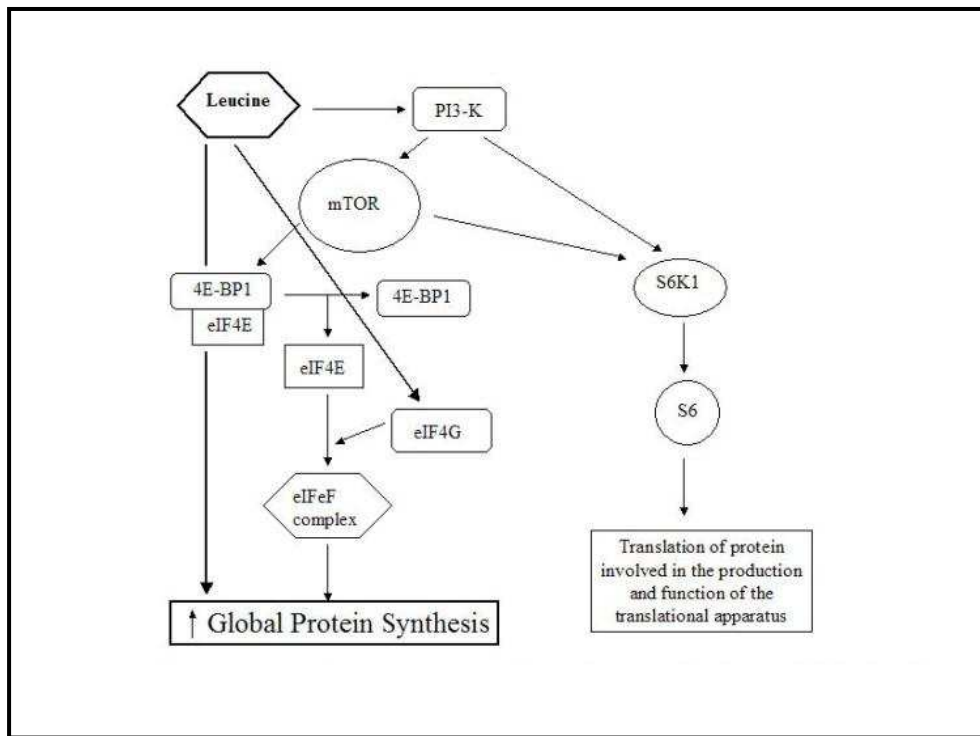


Figure 1. Mechanisms of initiation of protein synthesis (reprinted from E. Ha, MB Zemel/J Nutr Biochem 14 (2003) 251-258).

Anthony et al. (43) showed that leucine was unable to stimulate muscle protein synthesis in rats when rapamycin, a specific mTOR inhibitor was used. They also found administration of small meals containing either leucine or a combination of carbohydrate plus leucine to rats, resulted in increased rates of protein synthesis in rats (44). The fed rats had an increase in the association of eIF4E with eIF4G to form the eIF4F complex (44). Increases in serum insulin are not sufficient to enhance rates of translation initiation, and carbohydrate alone has no effect on 4E-BP1 phosphorylation or eIF4E availability (45). In contrast, leucine from dietary protein appears to stimulate the translation initiation by enhancing the formation of the active eIF4F complex.

Diabetic rats exhibit 35% of the rate of skeletal muscle protein synthesis of that observed in food-deprived, non-diabetic controls, but administration of leucine results in stimulation of protein synthesis by ~50%. However, that rate is well below that of the non-treated, non-diabetic controls (46). This shows that leucine works via both insulin-dependent and insulin-independent pathways to stimulate protein synthesis. A recent study by Macotela et al. (85) demonstrated that leucine could improve glucose intolerance and alter the development of type 2 diabetes in mice. In this study, mice were placed on a regular chow diet (CD) or a high-fat diet (HFD), with or without supplemental leucine. The results showed that after 8 weeks, the mice on the HFD had a 40% increase in their body weight compared to the CD, however the HFD + leucine mice had significantly smaller increase in subcutaneous fat mass than mice on the HFD alone. They also showed that the mice fed leucine had significantly improved glucose tolerance compared to the HFD, had improved insulin sensitivity and enhanced activation of the insulin receptors IRS-1 and AKT, and found that leucine supplementation caused insulin-stimulated p70S6K activation. Macotela et al. (85) found that the HFD increased glucose and glucose metabolites in muscle, which was normalized by leucine treatment. Fructose and sorbitol were also increased in the livers of mice on the HFD, which were returned to normal by the supplemental leucine. This study showed how one branched-chain amino acid that can only be obtained from dietary intake, leucine, could be a single environmental factor that influences multiple tissues and metabolic pathways and alters insulin signaling in a mouse model of insulin resistance and metabolic syndrome. A human study by Gannon et al. (22) showed that a high protein diet with high amounts of

dietary leucine, reduced glycemia in patients with type 2 diabetes and improved overall glucose control, a result of improved insulin secretion.

The insulin-independent (and mTOR-independent) pathway through which leucine increases protein synthesis may involve direct phosphorylation of eIF4G, however the mechanisms are unknown (47). Anthony et al. (49) used somatostatin, an inhibitor of pancreatic hormone release, in an attempt to maintain insulin concentration while administering leucine to rat skeletal muscle. They found the serum insulin concentrations were maintained at basal levels, preventing the phosphorylation of 4E-BP1 and S6K1, and somatostatin attenuated the leucine-induced changes in these mTOR components. However, somatostatin had no effect on the eIF4G*eIF4E assembly, showing the eIF4F complex occurs independently of increases in insulin (49). Another study looking at eIF4G phosphorylation used hindlimb preparations from post-absorptive rats and treated them with either food-deprived (1X) or super-physiologic (10X) concentrations of leucine. The results showed the stimulatory effects of leucine on protein synthesis was unaffected by an inhibitor of PI3-kinase and the phosphorylation states of 4E-BP1 and S6K1 were not further enhanced by 10X compared with 1X leucine. However, they did find that the binding of eIF4E to eIF4G was enhanced in the 10X leucine, showing leucine stimulates eIF4E*eIF4G assembly and protein synthesis directly in skeletal muscle, possibly through the phosphorylation of eIF4G through a signaling pathway independent of mTOR (50).

The role of leucine in post-exercise recovery has been the topic of many studies. Following exhaustive endurance exercise, the concentration of plasma leucine decreases dramatically (52). In a study using rats and treadmill running for 2 hours, Gautsch et al.

(52) found that rats experienced a 25% decrease in the rate of muscle protein synthesis, compared with the fasting controls receiving no exercise. The rats were provided a series of recovery drinks to determine the effect of carbohydrate and protein on muscle protein synthesis. The electrolyte drink contained glucose and sucrose only and was found to increase blood glucose and insulin concentrations, as well as muscle glycogen content, but did not have an effect on muscle protein synthesis. However, when provided a complete meal containing protein, or just leucine alone, the rats had complete recovery of muscle protein synthesis within the first hour after endurance exercise. This shows that leucine stimulates muscle protein synthesis through the mTOR pathway by activating eIF4E and S6K1. Other endurance exercise studies (53) have shown that following exhaustive endurance exercise, the mTOR pathway is inhibited, including inhibition of eIF4E and S6K1. Following endurance exercise, there is increased binding of eIF4E to the inhibitor 4E-BP1 and reduced binding of eIF4E to eIF4G to form the eIF4F complex (52, 53). It appears that the combination of leucine plus carbohydrates for recovery produces a synergistic effect, probably through the combined effects of leucine on mTOR and insulin on PI3-kinase (53).

Following resistance exercise, leucine ingestion plays a vital role in stimulating muscle protein synthesis. After resistance exercise, protein turnover is in a negative balance because protein breakdown is faster than protein synthesis. Dietary leucine is required to increase intracellular leucine concentration to activate mTOR and increase protein synthesis. When leucine is ingested at rest or when resistance exercise is performed in a fasting state, muscle protein synthesis is not nearly as high as when leucine is ingested following resistance exercise (54). Without leucine, muscles cannot

achieve maximum muscle protein synthesis and anabolic recovery. Dreyer et al. (55) conducted a study on male subjects and found that following resistance exercise and ingestion of essential amino acids (EAA) + carbohydrate (CHO), mTOR phosphorylation was significantly increased compared to controls who received no nutrients post-exercise. S6K1 and 4E-BP1 phosphorylation were significantly greater in the EAA+CHO group compared to controls, showing that muscle protein synthesis was greater in subjects consuming EAA (including leucine) and CHO than controls following resistance exercise. This study showed a 145% increase in the leucine-enriched EAA+CHO-induced mTOR muscle protein synthesis compared to only a 41% increase found in subjects just performing resistance exercise. Other studies have looked at the combined ingestion of CHO and EAA following resistance exercise. Koopman et al. (56) had three groups of subjects, who were randomly assigned to receive CHO, CHO+protein (PRO) or CHO+PRO+leucine following 45 minutes of resistance exercise. They found that plasma leucine concentrations strongly increased following ingestion of both CHO+PRO and CHO+PRO+leucine, whereas there was a slight decrease following the CHO trials. Whole body protein breakdown over a 6-hour recovery period was lower in the CHO+PRO and CHO+PRO+leucine trials compared to the CHO trial and whole body protein synthesis was increased in the CHO+PRO and CHO+PRO+leucine trials compared to the CHO trial (56). As with endurance exercise, the combined ingestion of protein and leucine with CHO following resistance exercise improves whole body protein balance during recovery.

Leucine has also been shown to improve endurance performance and upper body power following 6-weeks of supplementation. Crowe et al. (82) conducted a study on

outrigger canoeists. They tested upper body power and a row to exhaustion at 70-75% maximal aerobic power where perceived exertion (RPE), heart rate (HR) and plasma BCAA and tryptophan concentrations were assessed before and after 6-week supplementation of either leucine or placebo. Results showed that leucine supplementation resulted in significant increases in plasma leucine and total BCAA concentrations. They also showed that upper body power was significantly greater, rowing time significantly increased, and average RPE significantly decreased after leucine supplementation.

Fat Oxidation and Exercise

Many factors affect fat oxidation in exercising individuals. Exercise intensity, duration and type (anaerobic vs. aerobic), individual fitness level and previous training, gender, genetics, dietary intake prior to exercise (CHO vs. fat) and energy expended during exercise can all impact the amount and level of fat oxidation (57). Evidence suggests that moderate-intensity exercise yields the highest fat oxidation used for substrate in the average individual. However, it has also been shown that all intensities of exercise promote fat oxidation in the post-exercise period (57).

During exercise, the two main types of fuel that can be used are CHO and fat. The nature of the exercise bout and the training status of individuals will affect the utilization of these fuels. Low to moderate intensity exercise elicits greater fat oxidation in comparison to higher intensity or anaerobic exercise that elicits greater metabolism of CHO for fuel (57). This shift from fat-based to CHO-based fuels as exercise intensity increases, is known as the crossover point concept (62). For the power and speed sustained by athletes during training and competition, the primary source for fuel for the

muscles becomes CHO. However, the longer an exercise bout is sustained, the more fat oxidation contributes to total energy metabolism (63). Protein can also provide an alternative energy source during exercise. Several amino acids are used as fuel during exercise. Depending on the intensity and duration of exercise, amino acids can provide up to 10% of the total energy for sustained exercise (91). The largest use of protein for energy during exercise is in the glycogen-depleted state (97).

When measuring exercise intensity, researchers look at the percentage of maximum oxygen consumption (VO_{2max}) at which athletes or individuals are exercising. Fat oxidation rates peak around 64% of VO_{2max} , with maximum rates of 0.60 g/min in trained individuals (59). A similar study was done on moderately and highly trained individuals, and found maximal fat oxidation rates of 0.55 g/min to be at 63% of VO_{2max} (60). Once exercise intensities went above 65-70% of VO_{2max} , fat oxidation rates decreased significantly. This study by Achten et al. (60) then divided the test group into moderately and highly trained groups, and found maximal fat oxidation rates at 0.48 and 0.56 g/min respectively. The exercise intensities where these peak fat oxidation rates were observed did not significantly differ between the two groups. The effects of training will be discussed later on. Other studies have shown that fat oxidation accounts for up to 90% of total substrate utilization during exercise <50% of VO_{2max} (64). In comparison, high intensity exercise at 85% of VO_{2max} , resulted in CHO oxidation, specifically muscle glycogen instead of fat oxidation (57).

The effects of endurance training on fat oxidation have been extensively studied. Training reduces the reliance on CHO as an energy source, and increases fat oxidation during sub-maximal exercise (59). Friedlander et al. (65) conducted a study comparing

substrate use in trained and untrained women. $\text{VO}_{2\text{max}}$ was increased by 20% after a 12-week endurance training program and respiratory quotient (RQ) decreased significantly from 0.91 to 0.86 after training. Unlike endurance exercise, the effect of resistance training on substrate use has not been extensively studied. The studies that have been conducted focus on the fat oxidation rates in the hours immediately following exercise. RQs are significantly lower following resistance exercise in comparison with before the exercise bout (59), indicating increased fat oxidation. For example, Shuenke et al. (66) found that RQs were 0.89 in the 24 hours before the resistance exercise bout and 0.79 immediately following the 31-minute bout. Oxygen consumption measurements were obtained at consistent times (34 h pre-, 29 h pre-, 24 h pre-, 10 h pre-, 5 h pre-, immediately post-, 14 h post-, 19 h post-, 24 h post-, 38 h post-, 43 h post-, and 48 h post-exercise). Post-exercise measurements were compared to the baseline measurements made at the same time of day (66). Comparing the post-exercise fat metabolism of endurance and resistance exercise has provided contradicting results. Burleson et al. (67) showed that resistance exercise results in higher fat oxidation and lower RQs compared with aerobic exercise. In contrast, Melanson et al. (68) found similar post-exercise fat oxidation and RQ between the two types of exercise, with less energy expended during resistance exercise (322 kcals) than aerobic exercise (464 kcals). They concluded the difference might be due to the source of substrate used, with resistance exercise utilizing more CHO. Despite conflicting findings, training increases fat oxidation during sub-maximal exercise, whether resistance or endurance exercise (59).

Dietary CHO and fat can alter substrate utilization during exercise. There are numerous factors that affect fat oxidation such as the amount of fat, timing of the meal

and duration of the dietary treatment (57). Generally, the higher the fat content consumed before exercise, the higher the fat oxidation during and following exercise. Hawley et al. (69) conducted a study on trained cyclists and fed them a high-fat meal 90 minutes prior to an intense exercise bout. They found an increased rate of fat oxidation and fatty acid concentration during the exercise period (69). In contrast, it has been demonstrated that the ingestion of CHO before an exercise bout can result in decreased fat oxidation (59). The transition of muscle to higher reliance upon fat oxidation spares utilization of plasma glucose during fasting and delays consumption of muscle glycogen during exercise (64). The timing of the intake is an important factor. When a high-fat meal was delayed 90 vs. 240 minutes prior to exercise, there was a 2-fold decrease in plasma free fatty acids (57). And when CHO is ingested before the start of exercise, RQ is significantly higher than during exercise under fasted conditions (70). Insulin has an antilipolytic effect and even at low concentrations, insulin blunts lipolysis and enhances cellular uptake of CHO (59). It has been proposed that inability to increase reliance upon fat oxidation is related to the pathogenesis of insulin resistance in skeletal muscle (64). Ukropcova et al. (77) examined the capacity for fat oxidation by skeletal muscle and found that it was increased in subjects with increased insulin sensitivity, leanness, and aerobic fitness. Studies have also been performed using longer dietary treatments. Stepto et al. (71) showed that after only 3 days of consuming a diet containing 65% fat, RQ values dropped from 0.89 to 0.79 in a group of trained cyclists who exercised daily. In an even longer study in which untrained men consumed either a high-fat or high-CHO diet for 7 weeks, followed by 1 week of a high-CHO diet, the fat oxidation rates were significantly lower after 7 weeks of the high-fat diet (73). In addition, a randomized,

cross-over study, after 11 days of consuming a diet containing almost equal proportions of CHO and fat (45% CHO, 40% fat, and 15% protein), during which trained runners performed daily intense exercise, Achten et al. (72) showed fat oxidation rates were significantly increased and muscle glycogenolysis decreased compared to the 11-day high CHO trial.

Exercise increases the demand for energy and therefore causes macronutrient oxidation (57). There are numerous factors that can affect and regulate fat oxidation, but there is a considerable degree of intersubject variability in substrate utilization (59). Even when factors such as diet and training status have been controlled for, there is still a great deal of variation. Physical activity level, lean body mass, sex, VO_{2max} , and fat mass have been shown to account for only 34% of the variation in peak fat oxidation rates (74), leaving 66% of the variance related to dietary factors and genetics.

Leucine's Effect on Fat Oxidation and Mitochondrial Biogenesis

Research from this laboratory has shown that leucine promotes mitochondrial biogenesis, fatty acid oxidation, and energy partitioning to increase fatty acid utilization in muscle (17, 75). Leucine stimulated fatty acid oxidation in C2C12 mouse muscle cells in vitro and decreased fat storage and increased net fat breakdown in adipocytes. Further, leucine increased mitochondrial mass in C2C12 myocytes and increased the expression of mitochondrial regulatory genes (SIRT1, NRF, and PGC-1 α), as well as mitochondrial component genes (NADH dehydrogenase, cytochrome C oxidase, and uncoupling protein 3). Leucine increased mitochondrial mass and stimulated oxygen consumption, further reflecting an increase in mitochondrial biogenesis and fat oxidation in both cell types (75).

The same group recently investigated leucine's effects on fat oxidation in vivo in overweight and obese adults (18). They used a leucine-containing nutraceutical drink, which contained 2.25 g leucine, and was consumed in three daily doses of 0.75 g leucine/dose for 28 days. All subjects continued their usual diets and maintained pre-study physical activity levels throughout the study. Respiratory quotient was measured on Day 0 and Day 28 to assess fat oxidation through respiratory gas exchange. Sun and Zemel (18) found that the supplemented group exhibited a decrease in RQ and a corresponding increase in fat oxidation. They concluded that a nutraceutical drink containing leucine would effectively increase fat oxidation and possibly contribute to weight management in overweight and obese adults.

Materials and Methods

Research Design

This randomized experimental study used a crossover design as outlined in Figure 1. Subjects were randomized to receive either a leucine-supplemented beverage or a placebo beverage three times/day for seven days, followed by a seven-day washout period, and then followed by a final seven days on the remaining treatment (leucine or placebo). The primary independent variable was leucine supplementation and the primary dependent variable was respiratory quotient. Secondary outcomes included carbohydrate, protein, and fatty acid oxidation. This study was approved by the University of Tennessee Institutional Review Board prior to implementation.

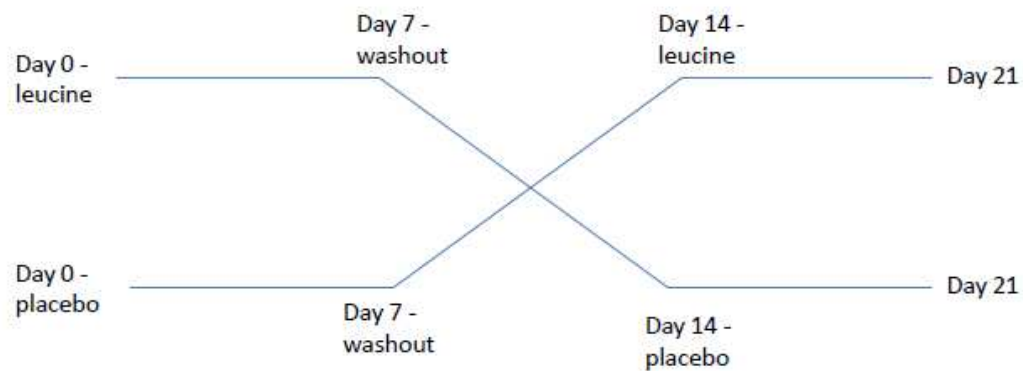


Figure 2. Cross-over design of study.

Study Population

This study was designed to determine the effects of leucine on fatty acid oxidation in a population of chronically active males, ages 25 to 55 years old, with BMI values of 18.5-27 kg/m². Subject characteristics are outlined in Table 1. Six men were recruited from Performance Training, Inc. (Knoxville, TN) and the student population of The University of Tennessee-Knoxville. Participants were recruited from flyers posted on the campus of The University of Tennessee, as well as through word of mouth to clients of Performance Training, Inc. Chronically active was defined as undergoing at least 3 hours (180 minutes) per week of vigorous-intensity physical activity, either muscle-strengthening or aerobic activity or a combination of both. Inclusion criteria for participants included:

- Age 25-55 years
- Male
- Body mass index (BMI) 18.5-27 kg/m²
- >180 minutes/week of vigorous-intensity physical activity

- Free of metabolic syndrome as defined by NCEP ATP III criteria, which includes the presence of three or more of the following risk determinants:
 - Abdominal obesity
 - Waist circumference >102 cm for males
 - Triglycerides >150 mg/dL
 - HDL cholesterol <40 mg/dL
 - Blood pressure >130 / >85 mm Hg
 - Fasting glucose >100 mg/dL

Exclusion criteria for participants included:

- BMI <18.5 kg/m² or >27 kg/m²
- Female
- <180 minutes/week of vigorous-intensity physical activity
- Use of medications for hypertension or high cholesterol
- Type 2 diabetes requiring use of any oral antidiabetic agent and / or insulin
- Presence of active gastrointestinal disorders such as malabsorption syndromes
- Chronic use of anti-inflammatory agents within the last four weeks
- Use of antioxidant supplements within the last four weeks
- Use of protein or other leucine-containing supplements within the last four weeks
- Recent (past 12-weeks) history of tobacco use
- Lactose intolerance and/or soy allergy

Participation in this study was determined based on self-reported data on a Medical Questionnaire (see Appendix). Inclusion and exclusion criteria questions, such

as whether subjects had high blood pressure, high cholesterol, high triglycerides or diabetes, were asked on the Medical Questionnaire and subjects were chosen for participation in the study based on their responses. Blood lipids were not measured, however. Blood pressure and waist circumference were measured in the clinic at the first meeting. These were not variables in the study or necessary measurements however both measurements are protocol for the Metabolic Clinic. Most participants in the study were approached at a fitness facility where they trained with Performance Training, Inc., and it was known prior to approaching them, whether or not they fit the exercise criteria for the study. However, the Medical Questionnaire contained questions on exercise habits, so participation was based on self-reported data for exercise as well.

Table 1. Baseline characteristics of subjects (mean \pm s.d.).

Variable	N = 5
Age (years)	37.4 \pm 11.3
Height (m)	1.79 \pm 0.07
Weight (kg)	82.1 \pm 6.9
BMI (kg/m ²)	25.2 \pm 1.3
Waist Circumference (cm)	83.9 \pm 6.3
Blood Pressure (mm Hg)	
Systolic	113 \pm 12.6
Diastolic	72.6 \pm 9.7
Resting Heart Rate (bpm)	65.4 \pm 10.3
Ethnicity	
Caucasian	4
African American	1
Exercise per week (minutes)	
Cardiovascular	206 \pm 77.0
Muscular Strengthening	156 \pm 93.4

Diet Assignment

Subjects were randomly assigned to either a placebo or supplement treatment. Nutritional supplementation consisted of a leucine-containing milk-based drink, *Just! Chocolate* (O-AT-KA Milk Products Cooperative, Inc., Batavia, NY), providing 0.75 g of leucine per dose. The supplement was taken three times daily with meals for 7 days, for a total leucine intake of 2.25 g per day from supplements. Each serving was 8 oz. The placebo was a drink of identical caloric value without the supplemental leucine. See Table 2 for the nutritional contributions from each drink. The placebo drink was 8th *Continent Light Chocolate Soy Milk*. Each subject was asked to maintain caffeine intake at a constant level for the entirety of the study. Each subject was asked to keep a 7-day diet record prior to the study. See Table 3 for the baseline dietary intake of the subjects contained in the 7-day food records. A food analysis program was used to analyze the food records with the nutrient data provided by the United States Department of Agriculture (USDA) (90). Diets were not prescribed, but subjects were asked to maintain normal dietary habits throughout the 21-day study and not replace any meals or snacks with the test beverages. Food logs were not kept throughout the duration of the study, however, so dietary intake could not be controlled for. Additionally, each subject recorded their physical activity and use of protein and leucine supplements in the 7-day diet log prior to the start of the study. Physical activity logs were not kept during the study, but each participant was asked to maintain normal physical activity levels for the duration of the study. If any participants were consuming leucine-containing supplements, they were unable to start the study until they had stopped taking their supplement for 1 month. This was also self-reported.

The compliance of all subjects was assessed by weekly beverage container return to determine whether greater than > 80% consumption of the beverage occurred.

Table 2. Energy and macronutrients provided by the test beverages.

Per 8 oz. serving	Just! Chocolate	8th Continent Light Chocolate Soy Milk
Calories (kcal)	90	90
Fat (g)	0	1.5
Carbohydrate (g)	16	12
Protein (g)	8	7
Leucine (g)	0.75	0.5

Table 3. Daily dietary intake of subjects prior to study (mean \pm s.d.).

Variable	N = 5
Calories (kcal)	2264.3 \pm 379.5
Fat (g)	78.9 \pm 21.2
Carbohydrate (g)	274.1 \pm 35.6
Protein (g)	107.3 \pm 24.5
Leucine (mg)	4683.2 \pm 1822.9
Leucine (mg/g protein)	79.7 \pm 8.9

Procedure

Each participant came to the Metabolic Clinic for an enrollment session, where they were screened for participation in the study. Height and weight were measured, BMI was calculated, and waist circumference, resting heart rate (RHR) and blood pressure were measured. Participants filled out a Medical Questionnaire and Informed

Consent Form (see Appendix). Based on the participants' responses on the Medical Questionnaire and their self-report of weekly physical activity via a 7-day physical activity log, they were deemed appropriate to participate in the study. Participants were given a 7-day food log to complete and bring back to their first session. They were also given instructions and containers for urine collection to be used the day before their first session. During the first session (Day 0), each participant came in after a 12-hour fast and 24-hour abstention from exercise. They provided 24 hours of collected urine for urea nitrogen analysis. Weight was recorded and a baseline resting metabolic rate (RMR) was obtained using the metabolic cart, as described below. Each participant was tested close to the same time, within one hour, each of the four testing days. Participants were fed a light breakfast, similar to one they normally consume based on their 7-day food record, following measurement of RMR. Participants were asked during the enrollment session what a typical breakfast was like, and based on response, they were provided fresh fruit, coffee and frozen breakfast burritos. Participants were also provided with the first 8 oz. of the appropriate (leucine or placebo) drink. Each participant was randomized prior to the start of the study to either supplement or placebo drink for the first 7 days. Participants were instructed to consume 3 - 8 oz servings of their drink every day for 7 days and were provided with 168 oz. of the appropriate drink, along with containers for urine collection. The second session on Day 7 was identical to the first, except this was a washout period and no drink was consumed for the subsequent week. The same measurements were taken on Day 14, and the other drink was provided and consumed for this week. Day 21 was the fourth and final session, where weight and resting metabolic rate were measured.

Resting Metabolic Rate/Substrate Oxidation

Resting metabolic rate (RMR), or resting energy expenditure (REE), is an estimation of how many calories are burned at rest. The use of indirect calorimetry is the standard procedure in research by which REE is measured. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) are used to calculate REE in accordance with the Weir formula (92). Respiratory quotient (RQ) is calculated from the ratio between expired carbon dioxide and consumed oxygen and supplies information on substrate utilization; for example, an RQ of 1.0 is indicative of glucose oxidation, 0.8 for protein oxidation and 0.7 for fat (88). As each of these substances is metabolized, they supply different amounts of heat and the total energy from oxidation can be computed by multiplying the number of grams of each metabolized substrate by certain factors; the factor customarily used for protein is 4.4 after deducting the potential energy in urine; for fat, 9.54; for carbohydrates, 4.19 (89).

From knowledge of the oxygen consumption and carbon dioxide production (l/min) and the urinary nitrogen (N, g/min; see Nitrogen Analysis below), one can calculate glucose (G), lipid (L), and protein (P) disappearance (DIS) rates (g/min) according to the following formulas (98):

$$\text{VO}_2 = 0.746 \text{ G}_{\text{DIS}} + 2.02 \text{ L}_{\text{DIS}} + 0.966 \text{ P}_{\text{DIS}}$$

$$\text{VCO}_2 = 0.746 \text{ G}_{\text{DIS}} + 1.43 \text{ L}_{\text{DIS}} + 0.744 \text{ P}_{\text{DIS}}$$

$$\text{N} = 0.160 \text{ P}_{\text{DIS}}$$

These equations can be solved for the respective substrates and rewritten as follows:

$$\text{G}_{\text{DIS}} = 4.57 \text{ VCO}_2 - 3.23 \text{ VO}_2 - 2.60 \text{ N}$$

$$L_{DIS} = 1.69 \text{ VO}_2 - 1.69 \text{ VCO}_2 - 2.03 \text{ N}$$

$$P_{DIS} = 6.25 \text{ N}$$

Nonprotein RQ (NPRQ) can also be calculated and is defined as the ratio between the nonprotein VCO_2 and the nonprotein VO_2 (the portion of the total VCO_2 and VO_2 that is attributable to carbohydrate and lipid disappearance):

$$\text{NPRQ} = \text{NPVCO}_2 / \text{NPVO}_2 = \text{VCO}_2 - 4.84 \text{ N} / \text{VO}_2 - 6.04 \text{ N}$$

Respiratory gas exchange was measured via the ventilated hood technique using a SensorMedics Vmax 29n metabolic cart (Sensor Medics, Anaheim, CA). All measures were obtained after a 12-hour fast and at least 24 hours abstention from exercise.

Following a urinary void, subjects rested quietly in the supine position for 60 minutes in a semi-darkened, thermoneutral (21-24 ° C) environment. Subjects had a clear, ventilated respiratory canopy placed over the head. The subjects remained in a quiet, supine position and breathed normally until steady state was achieved (normally 45 minutes).

Criteria for a valid resting metabolic rate (RMR) is a minimum of 15 minutes of steady state, as defined by <10% fluctuation in minute ventilation and oxygen consumption and <5% fluctuation in respiratory quotient (92, 95). Gas exchange was measured by an infrared CO_2 analyzer and an O_2 paramagnetic analyzer, and readings were taken for the steady state period for a determination of resting energy expenditure for a total of 30 minutes under these basal conditions for measurement of basal substrate respiratory quotient (RQ) and substrate oxidation. A 24-hour urine sample was collected for urea nitrogen analysis for each assessment in order to calculate nonprotein RQ and, subsequently, substrate oxidation (98). Quality control includes calibration of the gas analyzers before each measurement using unknown gases of 95% oxygen and 5% CO_2 .

Methanol burns were performed monthly to calibrate the flow rate and insure that the respiratory ratio and CO₂ recovery were within 2% of reference values.

Nitrogen Analysis

Protein is incompletely oxidized in the body and a portion of the protein molecule is excreted unburned in the form of urea (89). Since nitrogen is a major constituent of protein, measurement of nitrogenous metabolites in the urine reflects protein turnover in the body (96). Nitrogen balance can be estimated on the basis of the urine urea nitrogen excreted in a 24-hour period. A correction factor (4 g per 24 hours) is used to approximate insensible nonurea urinary nitrogen and fecal losses (88). With the 24-hour urinary nitrogen (UN, g/day), the complete Weir equation is used to adjust the calculation of REE for the incomplete oxidation of protein (99):

$$\text{REE} = [3.9 (\text{VO}_2) + 1.1 (\text{VCO}_2)] 1.44 - 2.17 (\text{UN})$$

Infinity Urea Liquid Stable Reagent (Thermo Fisher Scientific Inc., Middletown, VA) was used for the determination of urinary or urea nitrogen, which was then used to estimate total urinary nitrogen. The measurement of urinary urea nitrogen can be used to estimate total urinary nitrogen because about 80-90% of total urinary nitrogen is in the form of urea (96). The procedure utilizes a two-step enzymatic method, which utilizes urease to produce NH₃ from urea, followed by production of L-glutamate and NAD⁺ from the NH₃, α-ketoglutarate and NADH in the presence of glutamate dehydrogenase. The initial amount of urea present is proportional to the amount of NADH utilized in the second step of the reaction and is measured as the decline in absorbance at 340 nm.

Anthropometric Measurements

Body weight was measured with a calibrated scale and height measured with a wall-mounted stadiometer. Subjects were weighed at the beginning of each week, at the start of the session. Body mass index was calculated via standard equation (kg/m^2) using the body weight obtained during the enrollment session. Waist circumference was measured in the standing position, with measurements obtained midway between the lateral lower rib margin and iliac crest. The measurement was taken mid-exhalation, and the average of two readings was recorded. This measurement was taken only once prior to the start of the study.

Blood Pressure

Blood pressure and heart rate measurements were taken at the beginning of the enrollment session after the patient had been seated in an upright position in a chair for at least five minutes with the arm supported at heart level. Blood pressure was measured with an appropriately sized cuff using a standard sphygmomanometer on the same arm for every measurement. Three readings, at least one minute apart were taken and the average of the last two values reported. If these readings differed by more than 10 mm Hg (systolic or diastolic pressure), additional readings were taken until there were two successive determinations within 10 mm Hg of each other (93, 94). These blood pressure criteria are common practice for every study conducted in the Metabolic Clinic. This measurement was taken only once prior to the start of the study.

Statistical Analysis

Data were analyzed by repeated measures analysis of variance for each outcome, with baseline BMI and treatment order included as covariates in the statistical model.

Results

Six chronically active men completed this study. Five out of the six data sets were used in the data analysis due to perceived lack of compliance in one subject. Resting metabolic rates (RMR) were tested following a 12-hour fast. However, one subject had a respiratory quotient (RQ) in excess of 0.90, indicating recent carbohydrate intake. It was inferred that this RQ value was due to lack of compliance and consequently eliminated the data set. The results of this study are summarized in Table 4. There was a significant decrease in RQ in the leucine trial from Day 0 to Day 7 ($P = 0.000056$), whereas the control beverage exerted no effect on RQ. A decrease in RQ is indicative of increased fatty acid oxidation; although fat oxidation did increase by an average of 10.8 g/day in the leucine trial, this difference did not achieve statistical significance. Leucine exerted no significant effect on carbohydrate or protein oxidation. Resting energy expenditure (REE) was unchanged during the study. As seen in Table 4, protein oxidation during the placebo treatment had a large standard deviation in the Change: Day 7 – Day 0 column. This was due to an outlier, as one subject had a protein oxidation value that was twice the average. Also, REE during the placebo treatment had a large standard deviation in the Change: Day 7 – Day 0 column. This was due to wild swings of REE across the five participants.

Table 4. Effects of Leucine vs. Placebo Beverage on Indirect Calorimetry Parameters (mean \pm s.d.)

	Placebo			Significance
	Baseline	Day 7	Change: Day 7 - Day 0	Placebo Change
RQ	0.83 \pm 0.023	0.85 \pm 0.023	0.02 \pm 0.033	P=0.290 (NS)
Fat Oxidation (g/day)	48.8 \pm 26.28	49.6 \pm 9.71	0.8 \pm 29.76	P=0.955 (NS)
Carbohydrate Oxidation (g/day)	107.8 \pm 56.12	119.4 \pm 22.45	11.6 \pm 63.97	P=0.706 (NS)
Protein Oxidation (g/day)	96.4 \pm 68.41	82.4 \pm 25.64	-14 \pm 75.85	P=0.701 (NS)
REE (kcal/day)	1329.4 \pm 209.36	1327.8 \pm 78.06	-1.6 \pm 175.87	P=0.985 (NS)

	Leucine			Significance
	Baseline	Day 7	Change: Day 7 - Day 0	Leucine Change
RQ	0.85 \pm 0.032	0.81 \pm 0.032	-0.04 \pm 0.005	P=0.000056
Fat Oxidation (g/day)	48 \pm 13.19	58.8 \pm 20.14	10.8 \pm 23.04	P=0.354 (NS)
Carbohydrate Oxidation (g/day)	118.6 \pm 31.96	75.8 \pm 38.02	-42.8 \pm 34.84	P=0.052 (NS)
Protein Oxidation (g/day)	86.8 \pm 19.75	101.6 \pm 31.33	14.8 \pm 44.10	P=0.495 (NS)
REE (kcal/day)	1329.8 \pm 49.68	1316.4 \pm 151.52	-13.4 \pm 106.01	P=0.791 (NS)

RQ, respiratory quotient; REE, resting energy expenditure; NS, nonsignificant

Discussion

The major finding in this study is that leucine supplementation in chronically active males resulted in a decrease in RQ and it may increase in FA oxidation.

Leucine is a branched-chain amino acid that participates in numerous metabolic processes and is essential for muscle protein synthesis (81). It has been shown that

leucine is a modulator of muscle protein synthesis via the insulin-signaling pathway and that leucine stimulates muscle anabolism following exercise. It has also been suggested that leucine is ergogenic for both endurance and strength performance (82). However, there is little research on leucine's effect on FA oxidation. This study was conducted as a follow-up to research collected by our laboratory on the effect of leucine on FA oxidation in overweight and obese individuals. It was hypothesized that the same effects on fat oxidation would be found in chronically active individuals.

It has been found that the skeletal muscles of lean, aerobically fit individuals can dynamically switch between glucose and fat oxidation, however the skeletal muscles of sedentary, obese individuals are metabolically inflexible (64). Metabolic inflexibility is related to insulin sensitivity, percentage of body fat, and fitness. Active individuals can spare plasma glucose during fasting and delay consumption of muscle glycogen during exercise by relying on fat oxidation. Healthy, active individuals have a lower RQ during fasting conditions. Their skeletal muscles are more sensitive to insulin, causing a higher reliance on fat oxidation during exercise and the increase in energy demands and macronutrient oxidation to meet those demands during exercise, means an increase in FA oxidation both during and after exercise (57). It is possible that the chronically active men in this study already had a preference for FA oxidation in their skeletal muscle, therefore not increasing FA oxidation with leucine supplementation. Alternatively, it is possible that the diets of some of these subjects may have already had a high level of leucine at baseline. The RDA for dietary leucine is 16 mg/kg/day. The subjects in this study averaged 82 kg, meaning their recommended leucine intake is 1312 mg/day. From the 7-day food logs collected from each participant prior to the start of the study, subjects

averaged 4683 ± 1823 mg of leucine per day, which is 3.5 times more than what is recommended. Plasma levels of leucine from these subjects have recently been evaluated for a companion study. One subject had an elevated plasma leucine prior to supplementation and did not increase it with supplementation. Due to the small sample size of five subjects, we did not feel it appropriate to exclude this subject's data in our data analysis. However, for comparison purposes, the subject's data were excluded for analysis of fat oxidation and it was found that FA oxidation increased significantly by 19 g during the leucine trial compared with the placebo trial. Our original data analysis with this subject's data showed a 10.8 g increase in FA oxidation that failed to reach statistical significance.

In contrast, sedentary, obese individuals have a greater reliance on glucose oxidation than FA oxidation in fasting conditions. Body mass index, aerobic fitness and insulin sensitivity all affect skeletal muscles' ability to oxidize fat and dietary energy balance and training status modulate substrate-partitioning responses (64, 83). In a study of overweight and obese individuals, leucine supplementation significantly increased FA oxidation, while subsequently decreasing RQ. No significant changes were found in the placebo group (18). Another study on the role of leucine in weight loss showed that subjects consuming a high protein diet (and daily leucine intake of 5 g/day) had more weight loss than subjects consuming a control diet based on current dietary guidelines, although the difference was not statistically significant (81). The carbohydrate/protein ratio was 3.5 in the control diet and 1.5 in the high protein diet. Protein intakes were 0.8 g/kg/day and 1.5 g/kg/day respectively. In a second trial where the combined effects of diet plus exercise were studied, subjects in the high protein diet group lost significantly

more weight than subjects in the control diet group. Glisenzinski et al. (84) studied the effect of aerobic training on lipid utilization in overweight and untrained men and found that after 4 months of training, both lipid utilization and oxidation improved.

The effects of leucine on fat oxidation in lean, active individuals needs to be studied more in depth with greater participation. This was a small clinical trial ($n = 5$), which limited our data, however we did use a repeated baseline, repeated measures study design. The power of a statistical test is the probability that the test will reject the null hypothesis when the null hypothesis is actually false. A post-hoc power analysis was calculated, showing the results had 26% power and a minimum sample size of 13 subjects was needed for at least 80% power. Therefore, this study was not sufficiently powered to determine the effect of leucine on FA oxidation. We relied on self-reported data on exercise regimens and diet habits. Since diet records were not kept over the duration of the study, we were unable to see how intake and exercise habits may have changed over the course of the study, although subjects were instructed to keep their dietary and exercise habits the same. We were also unable to control for leucine intake during the course of the study. Subjects were instructed not to consume any other leucine-containing supplements during the course of the study, aside from the supplement drink, but they were not instructed to avoid high leucine foods. Blood was drawn from each subject about 90-minutes after the metabolic cart test and consumption of breakfast and the appropriate beverage. Plasma leucine levels were not analyzed for this study, but the results will be used to design and conduct future studies.

Conclusion

In conclusion, our results demonstrate that ingestion of leucine stimulates fat oxidation in chronically active men in comparison to a placebo. It is likely that active, fit individuals already have a lower RQ during fasting and their muscles have adapted to using fat as fuel, resulting in less significant fat oxidation results compared to an identical study conducted on overweight and obese, sedentary individuals (18). It is also a possibility that the diets of healthy, fit individuals contain high levels of leucine. Plasma leucine levels and dietary intake of leucine should be considered in future studies on both healthy, active and sedentary, overweight individuals.

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Appendices

Medical Questionnaire

Informed Consent

12. Do you have high triglyceride levels (≥ 150 mg/dL)?

Yes No Don't know

13. Have you been told by a doctor you have low HDL cholesterol (< 40 mg/dL)?

Yes No Don't know

Exercise Habits

1. Do you exercise? Yes No

2. If yes, answer the following:

a. What kind of exercise do you do? (Circle one)

Walking Jogging Aerobics Cycling

Weightlifting Other: _____

b. How many days a week do you exercise? _____

c. How many minutes do you exercise on the days you exercise? _____

d. When did you begin your exercise program? _____

Medication History

Please list all of the prescription medicines that you take:

Please list all over-the-counter medicines that you take (including antacids, pain relievers, cold/sinus medications):

Please list all vitamins, supplements (including protein supplements), and herbal medicines that you take:

Hospitalizations

Please list the year and reason for all times you were admitted to the hospital:

Please list any health problems that you have or have seen a physician for during the past 12 months

ID#: _____ (L/P or P/L)

INFORMED CONSENT STATEMENT
Effects of leucine on fatty acid oxidation in chronically active males
Summer 2009

INTRODUCTION

You are invited to participate in a research study designed to determine the effects of a nutrient supplement on the fatty acid oxidation in highly active and sedentary overweight individuals. Leucine may cause decreased energy storage in adipocytes, increasing fatty acid utilization in muscle. You have been invited to participate because you are either sedentary and overweight or chronically active. If you decide to participate in this study, you will participate in some tests (described below) and receive a drink to use three times a day with meals for seven days. The drink you receive may contain the active nutrient blend, leucine, or may contain chocolate soy milk. Following seven days, you will undergo a washout period for seven days where you will consume no drink and then consume the other drink for another seven days, three times a day with meals. You will not be able to choose which drink you receive first and will not know which one you received when until after the all volunteers have completed the study.

STUDY DETAILS

At the beginning of the study, you will be asked to keep detailed records of what you eat, your leucine supplement use and your activity level for 7 days. You will also have your blood pressure measured and your resting metabolic rate (RMR) tested. You must be fasting for 12 hours and have abstained from exercise for 24 hours for the RMR test. This test involves laying quietly in a comfortable chair for approximately 30 minutes while wearing a mask you're your nose and mouth; this permits us to measure how much oxygen you consume and how much carbon dioxide your produce. Blood tests will measure plasma leucine concentration, 1-hour post meal and supplement consumption. You will be provided with a small meal and your appropriate supplement drink prior to drawing blood.

The 21-day study begins at the completion of this first set of tests (day 0). You will then receive drinks to be used three times each day; these drinks will all contain the same ingredients, but some participants will receive a drink containing leucine and others will receive chocolate soy milk. You have an equal chance of being in either group for the first trial, and you cannot choose which group you are in. Blood and RMR tests will be repeated on days 7, 14, and 21. Day 7 is the end of the first trial and beginning of the second trial. The second trial is a washout, where participants do not consume any drink for 7 days. Day 14 marks the end of the second trial and beginning of the third, where participants will consume the drink that was not consumed in the first trial. Day 21 marks the end of the study.

RISKS

There is always a small risk of local bruising when blood is withdrawn from an arm vein. However, this is minimized by the use of trained medical staff and presents no significant danger.

BENEFITS

Obesity is a serious disease, resulting in over 300,000 preventable deaths each year in the U.S. The benefits of this study include identification of new approaches to assist overweight and obese individuals in successful weight control. You may experience improvements in your metabolic profile, although specific benefits cannot be guaranteed for any participating individual.

CONFIDENTIALITY

All study records related to your participation will be kept confidential. Data will be stored securely in a locked file and will be made available only to persons conducting the study unless you specifically give permission in writing to do otherwise. All data generated from this study will be pooled by group and, as such, no reference will be made in oral or written reports which could link any individual participant to the study. Data from this experiment will be retained for a period of seven years following completion of the study.

EMERGENCY MEDICAL TREATMENT

The University of Tennessee does not "automatically" reimburse subjects for medical claims or other compensation. If physical injury is suffered in the course of research, or for more information, please notify the investigator in charge (Michael B. Zemel, Ph.D. at 974-6238).

_____ Participant's initials

CONTACT INFORMATION

If you have any questions at any time about this study or the procedures, you may contact Michael B. Zemel, Ph.D. at The Department of Nutrition, 229 Jessie Harris Building on the UT Campus (1215 W. Cumberland Ave. Room 229). He may be reached by telephone at (865)-974-6238. If you have questions about your rights as a participant, contact the Compliance Section of the Office of Research at (865)-974-3466. The diet study office can be reached at (865)-974-6272.

PARTICIPATION

Your participation in this study is voluntary; you may decline to participate without penalty. If you decide to participate, you may withdraw from the study at anytime without penalty. If you withdraw from the study before data collection is completed your data will be returned to you or destroyed.

CONSENT

I have read the above information. I have received a copy of this form. I agree to participate in this study.

Participant's signature _____ Date _____

Investigator's signature _____ Date _____

Vita

Ciara Csanadi, RD, LDN received a Bachelor's degree in Biology with a minor in Chemistry from The Florida State University in December 2004, graduating Magna Cum Laude, with Honors in the Major. Ciara moved to Knoxville, TN in August 2006 and received a Bachelor's degree in Nutrition from The University of Tennessee, graduating Magna Cum Laude in May 2008. She was accepted into the combined Dietetic Internship – Masters Degree Program at The University of Tennessee and began her Master's coursework in August 2008. Ciara completed the Dietetic Internship in August 2010 and moved to Eugene, Oregon to take a position at The University of Oregon as the Sports Nutrition Intern. Ciara worked under the Sports Nutritionist, educating and counseling student-athletes, coordinating pre- and post-game meals, conducting body composition analysis, and managing the three nutrition bars available to student-athletes at the U of O. Ciara returned to Knoxville in April 2011 and passed the Registered Dietitian exam in June. Ciara currently works at Performance Training, Inc. in Knoxville as a Registered Dietitian and Personal Trainer.